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Phospholipids containing polyunsaturated fatty acyl groups are mitogenic for normal mouse mammary epithelial cells in serum-free primary cell culture

(phosphatidic acid/phosphatidylserine/saturated fatty acid/cyclic AMP)

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ABSTRACT **Epithelial cells obtained by collagenase digestion of mammary glands from virgin BALB/c mice were cultured in collagen gels in serum-free basal medium containing insulin (10 μg/ml), to which lipids or growth factors were added. Synthetic phospholipids were added as liposomes. Dilinoleoyl phosphatidic acid or phosphatidylserine or epidermal growth factor stimulated multifold growth. The optimum mitogenic effect of the phospholipids was dependent upon the presence of a polyunsaturated fatty acid esterified to the *sn*-2 position of the glycerol moiety. Dilinoleoyl phosphatidylcholine also stimulated growth but was generally less stimulatory than phosphatidylserine or phosphatidic acid, and phosphatidylethanolamine did not stimulate growth. Studies using phospholipids radiolabeled in either the *sn*-2 fatty acyl group or the glycerol backbone showed that the relative effect of phospholipids on growth did not correlate directly with the extent of their incorporation into cellular lipid, indicating that phospholipid turnover was the more important determinant for mitogenesis. Analysis of phosphatidic acid-stimulated growth suggested that both cAMP-dependent and cAMP-independent pathways were involved. Thus, mitogenic phospholipids stimulate proliferation by activating (directly or indirectly) multiple growth-regulatory pathways in mammary epithelial cells.**

When hormones or growth factors bind to their receptors, a set of early and late events is evoked that mediates the cellular response to these factors. In many cell types, the stimulation of proliferation by hormones or growth factors is coupled to an early rise in phospholipase (A_2, C) activity resulting in the generation of lipid and lipid-derived messengers that can activate a variety of growth-regulatory pathways (1, 2). These activities of lipids illuminate at least two distinct roles for lipids in cellular regulation: the known function of lipids serving a structural role as the building blocks of membranes and another function in which specific lipids play direct roles in transducing intra- or extracellular signals. That lipids may serve multiple roles in cellular function and regulation is intriguing for mammary epithelial biology, since these particular cells undergo a complex program of growth and morphogenesis within an adipose tissue matrix. Although the three-dimensional stromal matrix is required for mammogenesis, the adipose compartment itself may be capable of releasing fatty acids for utilization by the mammary epithelium (3) during growth and differentiation.

We have used a serum-free primary cell culture system to investigate the regulatory functions of lipids in mammary epithelial cell proliferation. Linoleic acid, by virtue of metabolism to its icosanoid derivatives (both E-series prostaglandins and hydroxy fatty acids), enhances the proliferation of mouse mammary epithelial cells in synergism with mammogenic hormones (progesterone and prolactin) and growth factors (refs. 4 and 5; W.I., unpublished observations). These lipids exhibit no growth-stimulatory activity in basal medium containing only insulin but can potentiate growth stimulated by another factor, such as epidermal growth factor (EGF). It appears that linoleate metabolites can activate growth-regulatory events that result in growth only when acting in synergy with events activated by another growth factor. In this sense, they are "incomplete" mitogens. In contrast to linoleate, we now find that specific exogenous phospholipids stimulate proliferation in mouse mammary epithelial cells in basal medium containing only insulin as a supplement. Thus, like growth factors or mammogenic hormones, and unlike linoleate, these phospholipids act as "complete" mitogens. This mitogenic effect of exogenous phospholipids suggests that phospholipid turnover in these cells can result in the activation of multiple growth-regulatory pathways and that phospholipid metabolism may play a crucial role in transducing growth-regulatory signals.

MATERIALS AND METHODS

Cell Culture. Mouse mammary epithelial cells were isolated by collagenase digestion (0.05%, CLS2, Worthington) of mammary glands from mature virgin BALB/c mice, followed by purification of epithelial cells by Percoll gradient centrifugation (see ref. 6 for details of the dissociation and culture procedures). For culture inside collagen gels, the cells were mixed with a neutralized, isosmotic rat tail collagen solution, and 0.5 ml containing 1–2 × 10^5 cells was pipetted into each well of a 24-well plate for growth studies. For radiolabeling studies, 2 ml of collagen solution containing 3–6 × 10^6 cells was pipetted into 6-well culture dishes. After gelation at room temperature, the gels were overlaid with the desired serum-free culture medium and incubated at 37°C in a 2% CO_2/98% air atmosphere. Cells were cultured for 10 days, except where indicated, after which the cultures, in triplicate, were terminated for DNA assay (7) or lipid extraction. The basal medium for all cultures was composed of a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium (F12/DMEM, GIBCO) buffered with sodium bicarbonate and 20 mM Hepes. This medium contained insulin (10 μg/ml), trace elements (6), soybean trypsin inhibitor (100 units/ml), and vitamin E (1 μg/ml). For cell culture on the surface of collagen-coated 6-well plates, the plates were prepared by covering the wells with a film of rat tail collagen (dissolved in acetic acid) and allowing the collagen to dry in

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Abbreviations: 16:0, palmitoyl; 18:1, oleoyl; 18:2, linoleoyl; 18:3, linolenoyl; 20:4, arachidonoyl; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; IBMX, 3-isobutyl-1-methylxanthine; EGF, epidermal growth factor.

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air. Cells were then seeded onto the plates in basal medium containing EGF (10 ng/ml, Collaborative Research), and bovine serum albumin (5 mg/ml, fraction V, Sigma). After the cells had spread, forming monolayers, the plates were washed with three changes of basal medium over 24 hr before test medium was added.

Liposome Preparation. All nonradiolabeled phospholipids were HPLC-purified synthetic phospholipids purchased from Avanti Biochemicals. They were stored at −80°C and their purity was confirmed by TLC. Dilinolenoyl phosphatidic acid [PA(di18:3)] and dioleoyl phosphatidylserine [PS(di18:1)] were prepared by phospholipase D digestion (8) of the corresponding phosphatidylcholine (PC). Liposomes were prepared according to Hamilton *et al.* (9). Aqueous lipid suspensions (in Puck's saline A) were passed three times through a French pressure cell at 18,000 psi (1 psi = 6.89 kPa). For growth studies, liposomes were prepared at a concentration of 2 mM (based on lipid phosphate) and contained 20% cholesterol (molar basis). Final lipid concentrations were determined by phosphate assay (10).

Preparation of [^{3}H]Glycerol-Labeled Phospholipids. [^{3}H]-Glycerol-labeled phospholipids were obtained by metabolic labeling of a rat tumor cell line. Cells were seeded at low density and cultured to confluency in 150-cm^2 flasks in serum-free basal medium supplemented with [^{3}H]glycerol (New England Nuclear, 200 Ci/mol; 1 Ci = 37 GBq) at 5 μCi/ml. The cells were harvested by mild trypsinization, washed, and stored at −80°C until lipid extraction.

The cells were extracted by the method of Folch *et al.* (11) by homogenization in chloroform/methanol, 2:1 (vol/vol). Phospholipids were obtained by fractionation of the lipid extract (dissolved in chloroform) on a silicic acid column (12) equilibrated with chloroform. The lipids were applied to the column, which was subsequently washed with 10 volumes of chloroform, 20 volumes of chloroform/methanol, 95:5 (vol/vol), and 20 volumes of methanol. The methanol fraction, containing the phospholipids, was fractionated by TLC (Sil G-60, EM Labs) in acid solvent (chloroform/acetone/methanol/acetic acid/water, 30:40:10:10:5, vol/vol) and the phospholipids were visualized by iodine staining. The PC, phosphatidylethanolamine (PE), and phosphatidylinositol (PI)/PS fractions were recovered by scraping the appropriate bands into glass tubes and extracting with chloroform/methanol, 1:2. The phospholipids were repurified by TLC in basic solvent (chloroform/methanol/28% ammonia, 65:25:5, vol/vol) and the purity was checked by TLC in acid solvent.

PS was separated from PI by TLC using plates prerun in 1.3% potassium oxalate in methanol/water, 2:1, and then dried (13). The TLC solvent was chloroform/acetone/methanol/acetic acid/water, 40:15:13:12:8. PS was recovered as described above. Specific activity was determined by scintillation counting and phosphate assay of the lipid fractions. PA was obtained by digestion of PC with cabbage phospholipase D (Sigma). The digestion was run to completion and PA was recovered by TLC in basic solvent followed by repurification in acid solvent.

Labeling Studies. Liposomes were prepared for studies comparing the uptake or diffusion of [^{3}H]glycerol-labeled PS, PE, PA, and PC by diluting the labeled phospholipids with their corresponding unlabeled dilinoleoyl (di18:2) species and then, for PS, PE, and PA, combining with an equimolar amount of carrier PC(di18:2). The lipids were suspended in F12/DMEM, converted into liposomes (see above), and added to cultures. The final medium composition was the same as basal medium above with the inclusion of liposomes. For cellular uptake studies, monolayer cultures were terminated by removing the incubation medium and washing the cells with medium 199. The cells were recovered by scraping and pelleting and then sonicated; an aliquot was removed for DNA assay, and the lipids were extracted by the method of Bligh and Dyer (14) as described (4).

Control experiments were done to compare the diffusion of liposomes into collagen gels. Radiolabeled liposomes were prepared as described above, then 0.2 ml of the liposome (2 mM) suspensions were overlaid on 0.5 ml of collagen gel (without cells) and incubated at 37°C. At various times, the liposome-containing medium was removed and the surface of the gels was rinsed quickly three times with 0.2 ml of F12/DMEM to remove residual medium. The last wash contained only 0.2% of the total medium radioactivity. Radioactivity remaining in the incubation medium and in the gel-entrapped medium were compared. If diffusion of liposomes to equilibrium throughout gel and medium were to occur, then 28% (2/7) of the radioactivity would remain in the medium, with 72% transferred to the gel. For different liposomes at 4 and 17 hr of incubation, the percent of radioactivity in the gel medium was as follows, respectively: PC-only, 38% and 50%; PC/PE, 35% and 45%; PC/PS, 43% and 61%; PC/PA, 39% and 52%; PA-only, 31% and 32%. All the phospholipids were able to enter the gels, although equilibrium was not achieved by 17 hr. Of all the liposome preparations, PC/PS liposomes diffuse at a slightly higher rate. PA-only liposomes, which precipitate in culture medium, diffuse relatively slowly. Thus, collagen gels do not constitute a selective diffusion barrier to these liposome preparations.

Fatty acid-labeled liposomes were prepared from mixtures of 2-[1-^{14}C]18:2-labeled PC or PA (58 Ci/mol, 0.03 μmol total) with 0.8 μmol of unlabeled PC(di18:2) or PA(di18:2), respectively. These liposomes were prepared in F12/DMEM and used in medium of the same composition as basal medium above. The final concentration of phospholipid in the medium was 0.02 mM. For these experiments, cells were cultured in collagen gels in basal medium containing PC/PA(di18:2) at 0.05 mM for 5 days. The medium was removed and the gels were washed with basal medium overnight before incubations with labeled liposomes. At termination, the cells in collagen gels were freed by dissolving the gels with acetic acid (0.1 volume of 0.25%) at 37°C and then recovered by centrifugation and washed three times with ice-cold medium 199. The cells were extracted and phospholipids were analyzed by TLC as described above. Neutral lipids were analyzed by TLC in hexane/diethyl ether/acetic acid, 70:30:1 (vol/vol).

RESULTS

Growth Studies. In order to define more precisely the phospholipid requirement for mitogenesis, a battery of synthetic phospholipids of defined fatty acyl composition were compared for their growth-promoting qualities in medium containing only insulin. Since in preliminary studies PA seemed capable of stimulating growth, it was tested alone and in combination with PCs of different fatty acyl composition. Fig. 1 shows the results of two similar experiments comparing PA(di18:2) and PC(di18:2). In experiment 2 the maximal growth response to PA was about half that of experiment 1. Overall, PA(di18:2) stimulated growth multifold (6- to 10-fold) over basal medium controls and was 2- to 3-fold more growth-stimulatory than PC(di18:2) despite aggregation and precipitation of PA(di18:2) liposomes in the culture medium. Because of this phenomenon, the dose–response curves between PC and PA may not be directly comparable, permitting only a qualitative comparison of responses at optimal concentration. In comparison, EGF stimulated growth 3- to 5-fold over basal medium controls. In further experiments, comparing phospholipids, carrier PC was always added to control more effectively the lipid concentration in the collagen gels, since diffusion studies (see *Materials and Methods*)

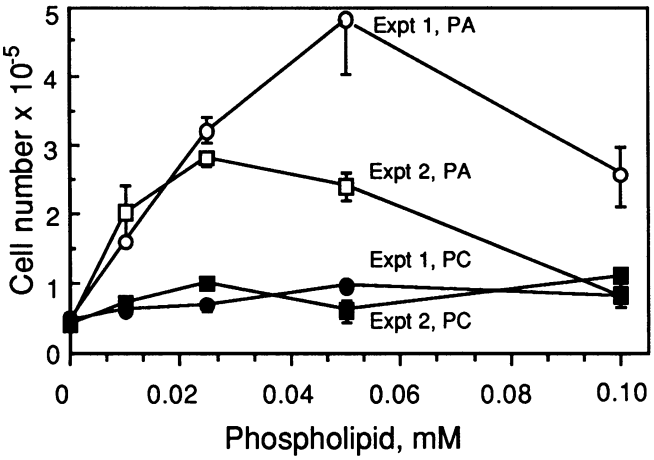

FIG. 1. Effect of PA(di18:2) and PC(di18:2) on cell proliferation. Cells were cultured in basal medium with or without phospholipid for 10 days. Growth stimulation is plotted as mean cell number and standard deviation of triplicates. Some standard deviations are smaller than the symbols. The results of two separate experiments are shown.

showed that the diffusion of these PC mixtures was similar. When PA(di18:2) was mixed with carrier PC(di18:2), forming stable liposome suspensions in culture medium, growth stimulation was again seen; however, the stimulation was about 3- to 4-fold, less than PA(di18:2) alone but still greater than PC(di18:2) only (Table 1, series A). Maximal growth-stimulatory concentrations ranged from 0.025 to 0.1 mM lipid phosphate among different experiments but were usually within 0.025–0.075 mM. At concentrations exceeding 0.1 mM, toxic effects could be seen resulting in cell degeneration within 10 days. In addition to PA and PC, other phospholipids were tested for their mitogenicity. PE(di18:2) and PS(di18:2) were each combined with PC(di18:2) in a 1:1 molar ratio and tested for their ability to stimulate proliferation. PE(di18:2) did not stimulate growth (data not shown), whereas PS(di18:2) stimulated growth to the same extent as PA(di18:2) (Table 1, series A).

These experiments showed that PA(di18:2) was a strong mitogen. The contribution of the esterified fatty acids of PA to this effect was next examined by preparing PAs of different fatty acyl composition [PA(1-16:0, 2-18:2), -(1-16:0, 2-20:4), and -(di18:3) were obtained by phospholipase D digestion of available synthetic PCs]. Table 1, series B, shows the results of four representative experiments comparing these PAs mixed with carrier PC(di18:2) in a 1:1 molar ratio. All were capable of stimulating growth, with PA(di18:2) and PA(di18:3)having the most consistent and greatest effect, although 18:3 (as the free fatty acid) does not stimulate the growth of mouse mammary epithelial cells (data not shown). PA(di18:1) stimulated growth (Table 1, series B, experiment 1), but the effect was inconsistent and generally less than that of PA(di18:2). In only 4 of 18 experiments did PA(di18:1) stimulate growth to the same extent as PA(di18:2). PCs of fatty acyl composition other than di18:2 were not tested alone. However, when PCs of different fatty acyl compositions (1-16:0, 2-20:4; 1-16:0, 2-18:1; or di18:2) were tested in combination with PA(di18:2) (1:1 molar ratio) the proliferative responses were equivalent (data not shown). Dose–response studies with PA and PC containing a saturated fatty acyl group [PC(di16:0)/PA(di16:0) or PC(di16:0)] showed that these phospholipids did not stimulate growth (Table 1, experiment 4). These results suggest that the minimal esterified fatty acid requirement for optimal mitogenesis is the presence of one polyunsaturated fatty acyl group at position 2 of the phospholipid's glycerol moiety.

Radiolabeling Studies. Since the relative effects of different liposomes on growth might be attributed to differences in incorporation and/or metabolism by the cells, these parameters were examined by comparing the ability of exogenous phospholipids to interact with cultured cells. Uptake was assessed by monitoring incorporation of [^{3}H]glycerol- or 2-[1-^{14}C]18:2-labeled phospholipids. The direct incorporation of [^{3}H]glycerol-labeled phospholipids delivered as liposomes was compared in cell monolayers to maximize the rate and extent of incorporation. When [^{3}H]glycerol-labeled PC, PS, PA, or PE was mixed with unlabeled PC(di18:2) (1:1 molar ratio) and equivalent concentrations of the phospholipid mixtures were added to cell monolayers, PC incorporation from the PC-only liposomes was greater than that of PS, PA, or PE mixed with PC(di18:2) (Fig. 2). For all liposomes, <16% of medium radioactivity was incorporated at 48 hr. For cells cultured in gels, the incorporation of radiolabeled PA or PC from liposomes of the same composition (PA/PC molar ratio 1:1) was comparable; i.e., incorporation of PC was only 50% higher than that of PA (data not shown).

Table 1. Effect of PC, PS, and PA of different fatty acyl compositions on cell proliferation

Phospholipid(s)*	Cell no. × 10^{-5}			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
		Series A		
No added lipid	1.1 ± 0.2	1.4 ± 0.3	1.1 ± 0	
PC(di18:2)	2.5 ± 1.2	4.6 ± 0.4	3.0 ± 0.5	
PC/PA(di18:2)	3.2 ± 0.4	6.5 ± 0.7	4.3 ± 0.1	
PC/PS(di18:2)	4.1 ± 0.1	6.5 ± 0.3	4.2 ± 0.2	
		Series B		
No added lipid	2.1 ± 0.2	2.3 ± 0.4	1.0 ± 0.3	1.4 ± 0.3
PC(di18:2)	5.7 ± 0.2	2.6 ± 0.2	3.4 ± 0.1	4.0 ± 0.6
PC(di16:0)				1.3 ± 0.3
PC(di16:0)/PA(di16:0)				1.3 ± 0.3
PC/PA(1-16:0, 2-18:2)	4.5 ± 1.1	4.4 ± 0.1	2.4 ± 0.1	
PC/PA(1-16:0, 2-20:4)	6.1 ± 0.2	5.4 ± 0.6	3.7 ± 1.0	
PC/PA(di18:3)	5.7 ± 0.5	6.1 ± 0.2	4.5 ± 0.4	
PC/PA(di18:2)	6.6 ± 0.2	6.5 ± 0.2	4.7 ± 0.1	6.5 ± 0.7
PC/PA(di18:1)	4.1 ± 1.1	2.6 ± 0.2	1.4 ± 0.6	

The results of three (series A) or four (series B) separate experiments, mean ± SD of three gels, are tabulated. PA or PS was added in combination with PC(di18:2) (1:1 molar ratio) except where indicated in experiment 4. Dose–response studies were done and the values shown represent the maximum growth achieved. For all experiments, the lipid concentrations producing maximal growth ranged from 0.025 to 0.075. mM.

*16:0, palmitoyl; 18:1, oleoyl; 18:2, linoleoyl; 18:3, linolenoyl; 20-4, arachidonoyl.

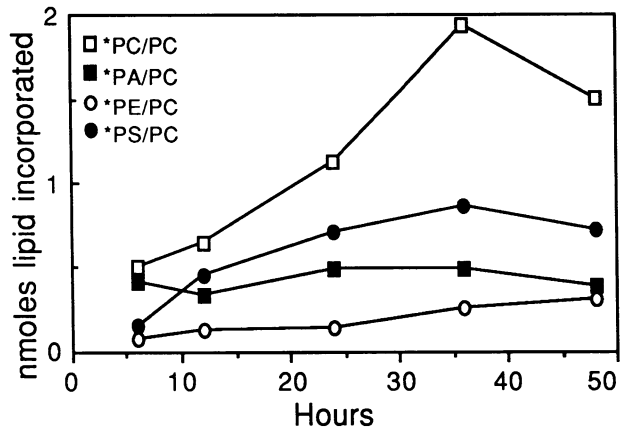

FIG. 2. Incorporation of exogenous [^{3}H]glycerol-labeled phospholipids into cellular lipid. Cell monolayers were incubated in the presence of liposomes (0.05 mM) composed of labeled PC, PS, PA, or PE mixed with unlabeled PC(di18:2) in a 1:1 molar ratio. Incorporation of labeled phospholipid from each liposome preparation is shown. Values are normalized to DNA and represent single determinations.

Since the fatty acyl composition of PA affects its mitogenic potential, the incorporation and deacylation of 2-[1-^{14}C]18:2-labeled PA or PC were examined. When the incorporation of the [^{14}C]18:2 moiety of these labeled phospholipids delivered alone was compared, cell-associated radioactivity derived from PA was higher than that from PC (17–23% vs. 10–15% of input radioactivity incorporated, respectively), but the cellular recovery of [^{14}C]18:2-labeled PA was less than that of [^{14}C]18:2-labeled PC (35–40% vs. 50–60% of radioactivity in cell lipid, respectively) after 6 hr of incubation. These results indicate a more rapid turnover of fatty acid esterified to PA than to PC when these phospholipids are delivered alone to the cells.

In summary, these results show that all exogenous phospholipids can be incorporated into cellular lipids with no strict correlation between the extent of uptake and mitogenicity. Rather, these results suggest that the metabolic fate of individual incorporated phospholipids may be a more important determinant of mitogenicity than the extent of cellular incorporation.

Phospholipids Stimulate Multiple Pathways. We previously showed (15) that linoleic acid (18:2) and its icosanoid derivatives raise intracellular cAMP levels in mouse mammary epithelial cells and that exogenous cAMP stimulates growth. If PA containing 18:2 (or 20:4) stimulates growth in part by causing an increase in adenylate cyclase activity, then growth stimulated by PA(di18:2) should be potentiated by the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine [IBMX, which alone does not stimulate growth or raise cAMP levels of cells in basal medium (15)]. Table 2 shows that IBMX potentiation did occur. Adenylate cyclase activity might be stimulated by prostaglandins synthesized from esterified linoleic acid (18:2) or arachidonic acid (20:4) released by phospholipases. In support of this concept was the observation that growth stimulation by PA(di18:2) was inhibited by a nontoxic concentration of indomethacin (10 μM; ref. 4), which inhibits prostaglandin synthesis from the linoleate metabolite arachidonic acid. The inhibition was only partial; PA(di18:2)-stimulated growth was inhibited by 48 ± 18% (range 23–72%, n = 6). The partial inhibition of growth by indomethacin, the PA(di18:2) synergism with optimal prostaglandin E_2 (itself not growth-stimulatory in basal medium) (Table 2), and the stimulatory effect of PA containing 18:3 instead of 18:2 suggest that the phospholipid activates both cAMP-dependent (IBMX synergism) and cAMP-independent pathways.

Table 2. Effect of PA(di18:2)/PC(di18:2) liposomes on growth in combination with IBMX and prostaglandin E_2 (PGE_2)

Addition(s)	Fold increase over basal
Liposomes	3.3 ± 0.8
Liposomes plus IBMX	6.8 ± 2.6*
Liposomes plus PGE_2	5.5 ± 1.2*

The results of five experiments are summarized. Data are expressed as fold increase (mean ± SE) in cell number over the cell number in cultures maintained in basal medium. Liposomes were added at 0.01–0.05 mM to medium with or without the indicated factors. The lipid concentration giving the maximal growth response was used to calculate the fold increase. IBMX (0.1 mM) and PGE_2 (10 μM) do not stimulate growth when added to basal medium alone. *Significantly different from liposomes only, paired t statistic, $P < 0.05$.

DISCUSSION

We previously showed that many factors, including EGF, fibroblast growth factor, mammogenic hormones, lithium, and phorbol esters, stimulate the proliferation of mouse mammary epithelial cells when added to serum-free basal medium containing insulin. Linoleic acid (18:2) alone does not stimulate proliferation in this basal medium but can potentiate the proliferative effect of these growth stimulators. This effect on linoleate is dependent upon its metabolism to icosanoids (4). We now find that exogenous phospholipids—unlike linoleate, and like mammogenic hormones or growth factors—can stimulate proliferation in the presence of insulin. This phospholipid-mediated mitogenic response is dependent upon both the nature of the head group and the fatty acyl composition of the phospholipid species. The effect of PA, PS, or PC containing an *sn*-2 polyunsaturated fatty acyl group is not simply a nonspecific, nutritional effect. We argue against this interpretation because the cells are capable of synthesizing all necessary phospholipids (4) and can undergo multifold growth stimulated by growth factors in the absence of exogenous phospholipid. Further, not all phospholipids are mitogenic, and those that are differ in their relative mitogenic capacities. Labeling studies suggest that simply the extent of uptake or incorporation of phospholipids is not a primary determinant of mitogenesis. The relative mitogenic effectiveness of the phospholipids seems to be influenced by the charge on the polar head group. Acidic phospholipids (PA and PS) seem to possess the strongest mitogenic activity. Soybean PI also can stimulate proliferation, although the effect is inconsistent (W.I., unpublished observations). Aside from considerations of differences in metabolism, this preference for acidic phospholipids may reflect differences in the cellular localization of these phospholipids. In the plasma membrane they are found predominantly in the inner leaflet, where they are accessible to interaction with cytoplasmic enzymes. In addition, PA (unlike PC and PE) is transported readily into intracellular membrane compartments (16) where it may assume a regulatory role. Other recent work has demonstrated that PA, but not PE, PS, or PC, is mitogenic for fibroblastic cell lines (17, 18). An unsaturated fatty acyl group was not required for optimal mitogenesis, however. In contrast, PA containing saturated fatty acyl group(s) does not stimulate the proliferation of mammary epithelial cells. In this respect, a stringent requirement for unsaturated fatty acyl groups in PA appears to be unique for mammary epithelial cells in this culture system.

The mechanisms by which PA and PS stimulate proliferation remain obscure, but the accumulated evidence suggests that multiple growth-regulatory pathways are activated. Aside from the activation of the cAMP/protein kinase A axis, PA may affect proliferation by the generation of diacylglycerol (17), leading to a sustained increase in this lipid inter-

mediate, which is known to activate protein kinase C (2). PC can also be a source of diacylglycerol (19), which may be significant since the PC pool is the largest among the phospholipids. Exogenous 1-oleoyl-2-acetylglycerol or short-chain diacylglycerol did not stimulate growth (W.I., unpublished observations); however, this was probably due to rapid deacylation by diacylglycerol lipase. In unpublished work (W.I.), it was found that incorporated [^{3}H]glycerol-labeled PA delivered from liposomes was only slowly metabolized and yielded elevated levels of diacylglycerol. PA has been reported to stimulate the hydrolysis of inositolphospholipids and to increase oncogene expression (17), but the significance of these pathways for normal mammary epithelial cells is unknown. Recently it was reported that arachidonic acid and some phospholipids can inhibit the GTPase-activating protein (GAP), suggesting that these lipids can potentiate Ras functions (20).

In addition, some species of protein kinase C can utilize PS (2) or unsaturated fatty acids (21, 22) as cofactors. Further studies examining diacylglycerol metabolism, as well as an assessment of protein kinase C activity, are needed before the significance of this pathway can be evaluated.

The growth *in vitro* of many cell types, particularly cell lines, is not dependent upon the availability of exogenous lipids (23). Similarly, the proliferation of normal mammary epithelial cells in primary culture is not absolutely dependent upon exogenous lipids; nevertheless, these cells are highly responsive to linoleate and specific phospholipids. In addition, the hormonal requirements for the differentiation of mouse mammary epithelial cells *in vitro* can be modulated by linoleate (24). These findings lead us to suggest that these epithelial cells, which grow and differentiate within an adipose tissue matrix, may utilize exogenous fatty acids supplied by the surrounding adipose cells during mammogenesis (4, 24).

Although phospholipids are available from the blood, a physiological role for these exogenous lipids in regulating mammary epithelial cell proliferation seems unlikely. Rather, we suggest that the effect of exogenous phospholipids on mammary epithelial cell proliferation *in vitro* shows that intracellular lipid-dependent pathways may be of primary importance in regulating the growth and differentiation of these cells. We envision that hormones and growth factors may stimulate phospholipid turnover, producing lipid metabolites that could then act as second messengers initiating cellular events regulating the differentiation and growth of these cells.

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